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Thiocolchicine dimers: a novel class of topoisomerase-I inhibitors

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Abstract

During a cellular screening of thiocolchicine analogs, thiocolchicine dimers resulted particularly active in cisplatin-resistant A2780-CIS cells. In order to discover by which mechanism(s) thiocolchicine dimers overcame cisplatin resistance, p53, $p21^{waf1}$ and MLH1 were assessed by Western blot. Results pointed out that, when combined with cisplatin, dimers increased the amount of all the three proteins with respect to the levels obtained by single drug exposure, thereby suggesting an interference in the process of repair of the cisplatin-induced DNA lesions. Moreover, in isolated nuclei drugs were able to produce DNA breaks, as demonstrated by Comet assay, thereby proving that the compounds were able to target cell nucleus independently from microtubules. Since Topo-I (topoisomerase I) is directly involved in the DNA repair and such activity is overexpressed in cisplatin-resistant cells, Topo-I was investigated as a potential target. Using DNA relaxation assay, thiocolchicine dimers inhibited Topo-I, a property not shared by thiocolchicine. At variance with camptothecin, dimers did not produce cleavable complexes, thereby indicating that Topo-I inhibition occurs upstream of the religation step. To assess the mechanism of inhibition, an electrophoretic mobility shift assay between DNA and Topo-I was performed and revealed that thiocolchicine dimers specifically interfere with binding of Topo-I to DNA. The interference is specific since the same compounds did not modulate DNase activity and did not act as intercalating agents in the DNA unwinding assay. Finally, behaviour of dimers as spindle poisons was investigated and no relevant changes with respect to thiocolchicine in terms of interaction with microtubules were found.

Keywords: Thiocolchicine; Topoisomerase-I; Drug-resistance; Spindle poisons; DNA repair; Cisplatin

1. Introduction

Colchicine is a plant alkaloid derived from autumn crocus (*Colchicum autumnale*) and is still one of the most effective treatments to relieve the intense pain associated with an acute gout attack. Such a potent anti-inflammatory activity is probably dependent on the fact that colchicine is able to inhibit microtubule dynamics, by hampering microtubule polymerisation [1]. The consequent microtubule paralysis blocks the release of pro-inflammatory mediators in leukocytes and other inflammatory cells [2].

In cancer cells microtubule-interacting drugs act as spindle poisons, thereby blocking the cell cycle at the M phase and inducing apoptosis [3]. This latter feature underlies the antitumour properties belonging to such agents. Therefore, colchicine and its active analogues exhibit excellent antitumour properties "in vitro" and have been extensively investigated as potential anticancer drugs. However, neither colchicine nor its analogues have been extensively used as anticancer drugs, due to the extreme toxicities and the consequent unfavourable therapeutic index noticed in preclinical experimental models and in clinical trials. Colcemid (*N*-deacetyl-*N*-methylcolchicine) was the unique colchicine analogue used as anticancer drug, but was early replaced by Vinca alkaloids, which exhibited a superior therapeutic index in clinical studies.

Colchicine framework (Fig. 1) includes a trimethoxyphenyl (A ring), a seven membered ring (B ring) with an acetamide at the seven positions, and a tropolonic group (C ring). Thiocolchine (Fig. 1) differs from colchicine owing to the presence of a methylthio group instead of a methoxy at C10 in the tropolonic ring. In earlier studies,

Abbreviations: EMSA, electrophretic mobility shift assay; Topo-I, topoisomerase-I; SSB, single strand break; NER, nucleotide excision repair; CPT, camptothecin

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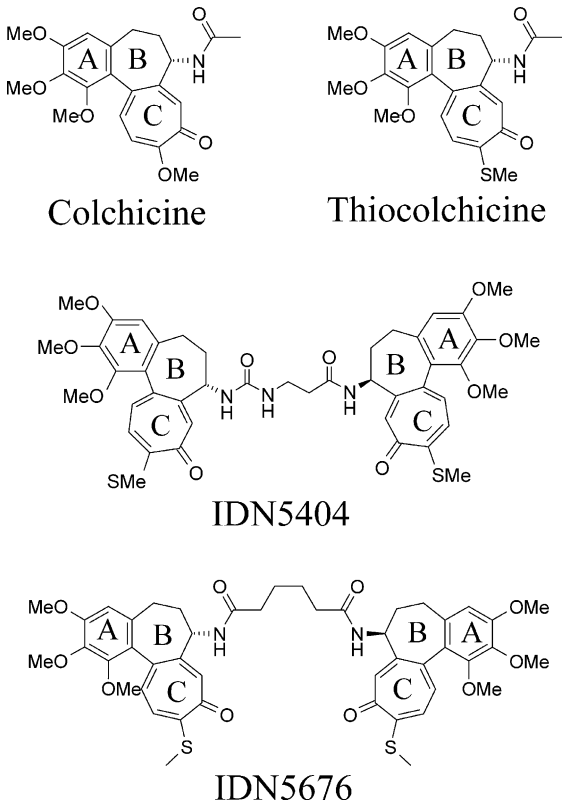

Fig. 1. Chemical structure of colchicine, thiocolchicine and thiocolchicine dimers. (A), (B) and (C) represent the three rings structure of colchicine.

we investigated the antitumour activity of a panel of thiocolchine analogues [4–6]. As a continuation of these previous activities, we focused our screening on a human ovarian cancer cell line (A2780wt) and its counterpart resistant to cisplatin (A2780-CIS). This screening strategy was adopted in order to identify newly developed analogues able to overcome cisplatin resistance. We discovered that thiocolchicine dimers, with an appropriate linker sequence, are particularly active in A2780-CIS. It is worth noting that such activity is connected to the inhibition of Topo-I, without loss of the spindle poison properties typical of the colchicine analogues.

2. Materials and methods

2.1. Antibodies and reagents

Compounds were supplied by Indena S.p.A. and diluted in absolute DMSO. These solutions were further diluted on each experimental day in order to achieve a 0.1% final DMSO concentration. Monoclonal antibodies anti $p21^{waf1}$, p53 (pAb240), hMSH2, GTBP and MLH-1 were purchased from Santa Cruz Biotechnology Inc. All the antibodies were finally diluted at 1:500. Secondary peroxidase-conjugated antibodies (BioRad) were used at the final dilution of 1:2000. Detection method was the Enhanced Chemoluminescence (ECL) from Amersham Biosciences on AXR5 films (Eastman Kodak). Topo-I enzyme was purchased from Topogen. All the other reagents were purchased from Sigma, if not otherwise specified.

2.2. Cell cultures

A2780 and A2780-CIS cells were purchased from the European Collection of Cell Cultures (ECACC). Culture media were selected according to ECACC suggestions. A2780 cells resistant to topotecan, the most commonly Topo-I inhibitor used in clinics, (A2780-TOP1 and A2780-TOP2) were generated in our laboratory after continuous exposure to stepwise increasing drug concentrations. Topotecan was supplied by GlaxoSmithKline.

To perform growth experiments cells were seeded (20,000 cells/well) in 96-well flat bottom plates (Cultureplates, Perkin-Elmer Life Science). After 24 h the media were replaced and, after one washing, media containing the drugs were added. Three independent experiments were performed in quadruplicates. After 72 h of culture in the presence of the tested compounds, plates were harvested and the number of viable cells was estimated by dosing ATP, using the ATPlite kit (Perkin-Elmer Life Science) and the automated luminometer Topcount (Perkin-Elmer Life Science). The kit was employed according to the manufacturer's suggestions. For each drug/cell line a dose–response curve was plotted and the IC_{50} values were then calculated by fitting the concentration–effect curve data obtained in the three experiments with the sigmoid-Emax model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect [7].

2.3. Nuclear isolation

After drug treatment cells were collected after trypsinisation and pelleted. After two washings in PBS, pellets were resuspended in a hypotonic solution for the nuclear isolation medium (NIM: 5 mM Tris–HCl pH 7.4, KCl 5 mM, $MgCl_2$ 1.5 mM, EGTA 0.1 mM, Triton X-100 0.2%) and incubated for 30 min. Then, cell nuclei were centrifuged at 4 °C and again incubated on ice with NIM. After one washing, pellets were resuspended in a nuclear lysis solution (NaCl 150 mM, Tris–HCl pH 7.4 10 mM, EDTA 5 mM, Triton X-100 1%). Protein concentration of the nuclear lysates was determined spectrophotometrically with the Bradford assay, using bovine serum albumine as internal standard.

2.4. Western blots

Fifty micrograms of nuclear proteins were denatured using 5× Laemli buffer (60 mM Tris–HCl pH 6.8, 25% glicerolo, 2% SDS, 0.715 M β-mercaptoethanol, 0.1% bromophenol blu) at 100 °C for 10 min. After denaturation, lysates were run in 12% polyacrilamide gels for

60 min at stable 50 mA. After electrophoresis proteins were blotted on PVDF membranes (90 min, 400 mA), and specific sites were saturated with 5% non-fat dry milk in 1× TBST (20 mM Tris–HCl pH 7.4, 137 mM NaCl, 0.1% Tween-20) for 60 min at room temperature. Then, primary antibodies were added and incubation lasted 18 h at 4 °C under constant shaking. After extensive washings in TBST, membranes were incubated for 2 h with secondary antibodies at RT and specific signals were detected through ECL, according to the manufacturer's suggestions.

2.5. Single cell gel electrophoresis (Comet assay)

Nuclei were isolated from A2780wt cells by incubating whole cells in a buffer containing 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 150 mM NaCl for 20 min on ice with gentle rocking. Plasma membrane disruption and nuclei integrity were checked under the microscope. Isolated nuclei were exposed to the drugs at 10 μM for 30 min at 37 °C or to H_2O_2 for 5 min. DNA breaks were detected as previously described [8]. Briefly, nuclei were embedded in agarose gel and then spread on a polylysinated microscope slide. Nuclei were lysed in 2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na_2EDTA, 1% Triton, 10% DMSO, pH 10, for 1 h at 4 °C. After lysis, nuclei were preincubated for 20 min at 4 °C in the electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13.5) and then subjected to alkaline gel electrophoresis (300 mA, 4 °C, 20 min). Nuclear DNA was stained using SYBR-gold (Molecular Probes). Slides were analysed by automatic image analysis (Casys system, Synoptics Ltd.) to quantitate DNA damage. The tail moment, calculated by multiplying the total intensity of the comet tail by the migration distance from the center of the comet head, was used to measure DNA damage. Fifty nuclei for each experimental point were scored blind from two slides.

2.6. Relaxation activity of topoisomerase-I

Topo-I functional activity was assayed by relaxation of supercoiled plasmid DNA in a cell free system. Briefly, relaxation of 500 ng of supercoiled DNA pBlueScript (Stratagene) by Topo-I (4 U) was performed in 30 μl of topoisomerase I relaxation buffer (20 mM Tris pH 7.5, 0.1 mM Na_2EDTA, 10 mM $MgCl_2$, 50 μg/ml acetylated-BSA, 100 mM KCl) in the presence or absence of the test compounds dissolved in DMSO solutions. Reactions were started by the addition of DNA. Control groups were either DNA alone or DNA treated with Topo-I. At different time points reactions were stopped by adding 0.5% (w/v) SDS. Products were then run in a 1% agarose gel in 50 mM Tris-borate (pH 8.3), 1 mM EDTA at 3.5 V/cm. Gels were stained with ethidium bromide and visualised with a UV transilluminator. Images were acquired and quantified through the Phoretix 1D software package (Phoretix International Ltd.).

2.7. Measurement of DNase I activity

Bovine DNase I (0.4, 2.0, 4.0 U/ml) was incubated with 400 ng of pBR322 DNA in 20 ml of buffer (50 mM Tris–HCl, pH 7.5, 10 mM $MnCl_2$, and 50 mg/ml BSA) in the presence of IDN5404 (up to 100 μM) for 15 min at 37 °C. The reaction was stopped by the addition of 25 mM EDTA (final concentration) followed by agarose gel electrophoresis, as described above.

2.8. Measurement of Topo-I mediated DNA cleavage

Reaction mixtures contained an excess of enzymes (i.e., 100 U of Topo-I). Samples, which contained tested drugs, were assembled in this order: drug, Topo-I, CPT. Reactions were started by the addition of DNA and terminated with prewarmed SDS [1% (w/v) final concentration]. After digestion with proteinase K, open circular and linear DNA were separated from intact supercoiled and relaxed form by agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide under the same conditions as for the relaxation assay.

2.9. Analysis of Topo-I/DNA-binding by EMSA

EMSAs were basically performed as described elsewhere [9]. In brief, supercoiled pBR322 DNA (Topogen) was incubated in 20 ml of relaxation topoisomerase I buffer with or without excess of topoisomerase I (100 U) in the presence of the test compounds at 37 °C for 6 min. The reaction was started by addition of DNA. The samples containing test compounds were assembled in the order compound, Topo-I, CPT. Samples were immediately loaded onto the 1% agarose gel in Tris-acetate-EDTA buffer with 1 μg/ml ethidium bromide and separated by electrophoresis for 6 h at 2 V/cm.

2.10. Measurement of DNA intercalation

Intercalation was determined by the unwinding assay [10]. Supercoiled pBR322 DNA was relaxed with 300 U of Topo-I at 37 °C for 15 min in Topo-I relaxation buffer. To confirm full relaxation of DNA, one sample was terminated with SDS after 15 min. Inhibitors were added and the incubations were continued for another 60 min. Parallel experiments ensured that Topo-I retained its activity in the presence of the compounds used. The reaction was terminated by addition of 1% (w/v) SDS and followed by digestion with proteinase K as described above. The compounds were removed by extraction with chloroform/isoamyl alcohol (24:1). For a better resolution of topoisomers, DNA was separated on 1% agarose Trisphosphate-EDTA buffer (36 mM Tris–HCl, pH 7.8, 1 mM EDTA, and 30 mM NaH_2PO_4) for 15 h at 0.4 V/cm. The gel was stained with ethidium bromide and acquired as described above.

3. Results

In order to obtain novel drugs able to overcome cisplatin resistance, a panel of thiocolchicine analogues were tested in both A2780wt and A2780-CIS, resistant to cisplatin. The IC_{50} values (drug concentration able to half-maximally inhibit cell growth of cancer cells) served to score the growth inhibition effects in cancer cells. IC_{50} values of cisplatin were 316 ± 119 and 7896 ± 2305 in A2780wt and A2780-CIS, respectively, and the resistance index (ratio IC_{50} resistant cells over IC_{50} parental cells) was nearly 25. The thiocolchicine dimers IDN5404 and IDN5676 yielded in A2780wt IC_{50} values of 18.6 ± 4.2 and 5.2 ± 0.7, respectively, whereas in A2780-CIS IC_{50} values were 1.4 ± 0.8 and 3.3 ± 1.1, for IDN5404 and IDN5676, respectively. The correspondent resistance indexes were 0.08 and 1.19 for IDN5404 and IDN5676, respectively. These findings pointed out that both thiocolchicine dimers were particularly active in cisplatin-resistant A2780-CIS cells.

As a first approach, we tried to gain further insights into the mechanisms of this increased activity in cisplatin-resistant cells. For this reason, we selected IDN5404 (Fig. 1), the thiocolchicine dimer exhibiting the highest increase of activity in A2780-CIS cells. By Western blot analysis the ability of the dimer to modulate the nuclear level of p53 (Fig. 2A) and its downstream effectors $p21^{waf1}$ (Fig. 2B) was assessed. Such an assay was performed in A2780wt and A2780-CIS cells. Equipotent doses (IC_{70}) of IDN5404 and cisplatin were used as single agents or in combination (i.e., 500 nM cisplatin and 30 nM IDN5404, 10,000 nM cisplatin and 2 nM IDN5404 for A2780wt and A2780-CIS, respectively). In A2780wt cells as compared to control cells cisplatin treatment was able to increase the expression of p53, whereas the combined treatment IDN5404/cisplatin further raised the p53 expression. Moreover, $p21^{waf1}$ was upregulated by both drugs as single agents and again the combined treatment further augmented its expression. On the other hand, in A2780-CIS only the combined treatment IDN5404/cisplatin was able to increase p53 levels, without affecting $p21^{waf1}$, thereby indicating that cisplatin resistance was associated to the disruption in such a cell line of control of p53 on the $p21^{waf1}$ pathway. Taken altogether these findings suggest that IDN5404 increased the genotoxic effects of cisplatin, as indirectly evaluated by the augmented levels of p53 and $p21^{waf1}$ (only in A2780wt) visible when the two drugs were used together. Therefore, it appeared likely that thiocolchicine dimers could interfere in the process of DNA repair in wild type as well as in drug-resistant cells. In order to

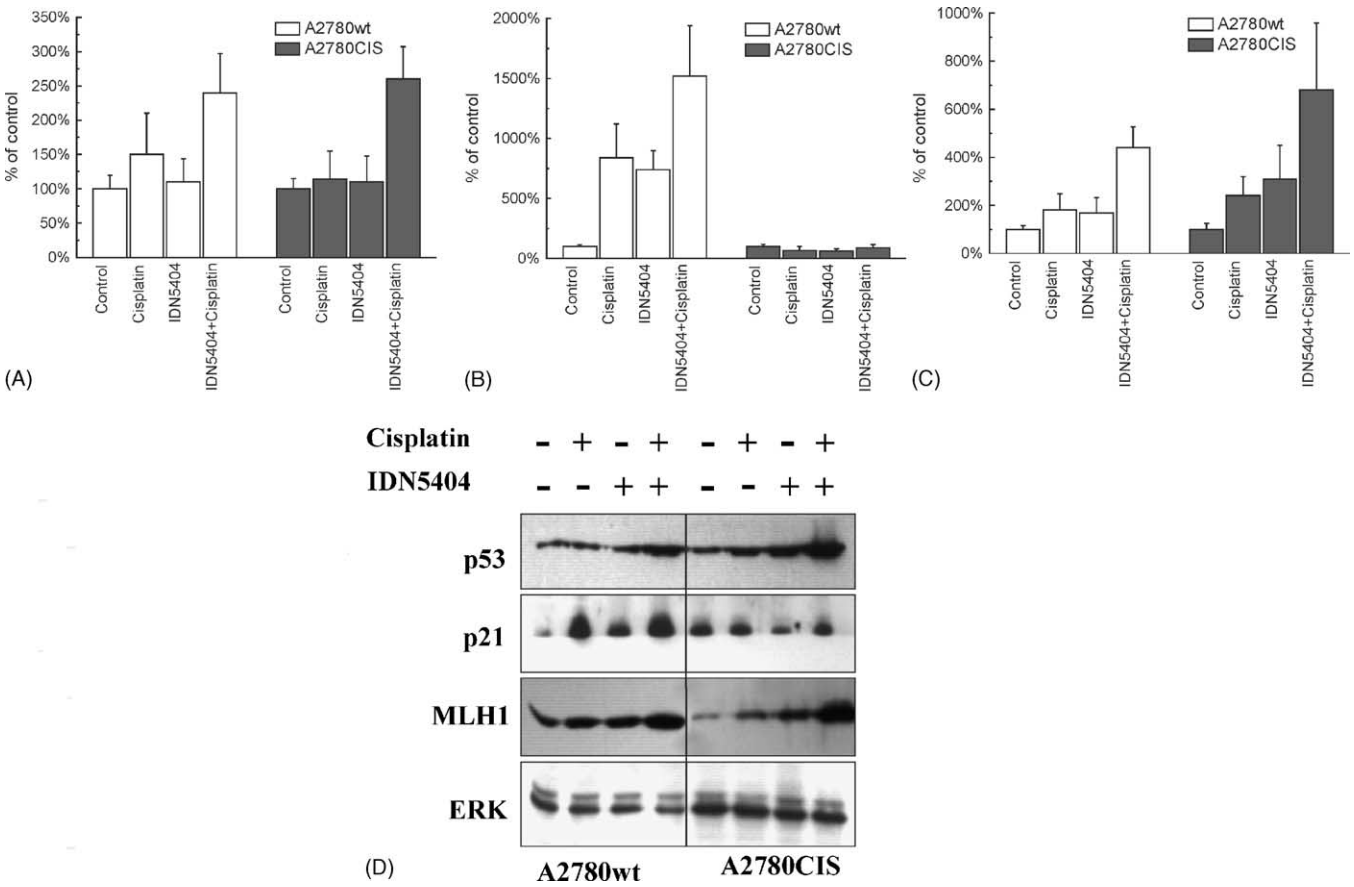

Fig. 2. Bar charts showing results of Western blot analysis for p53 (A), $p21^{waf1}$ (B) and MLH1. Open and closed bars illustrate data for A2780wt and A2780-CIS, respectively. Bars and error bars are mean and S.D. of three independent experiments. In (D) representative Western blots were reported. ERK was used to normalise expression for sample loading.

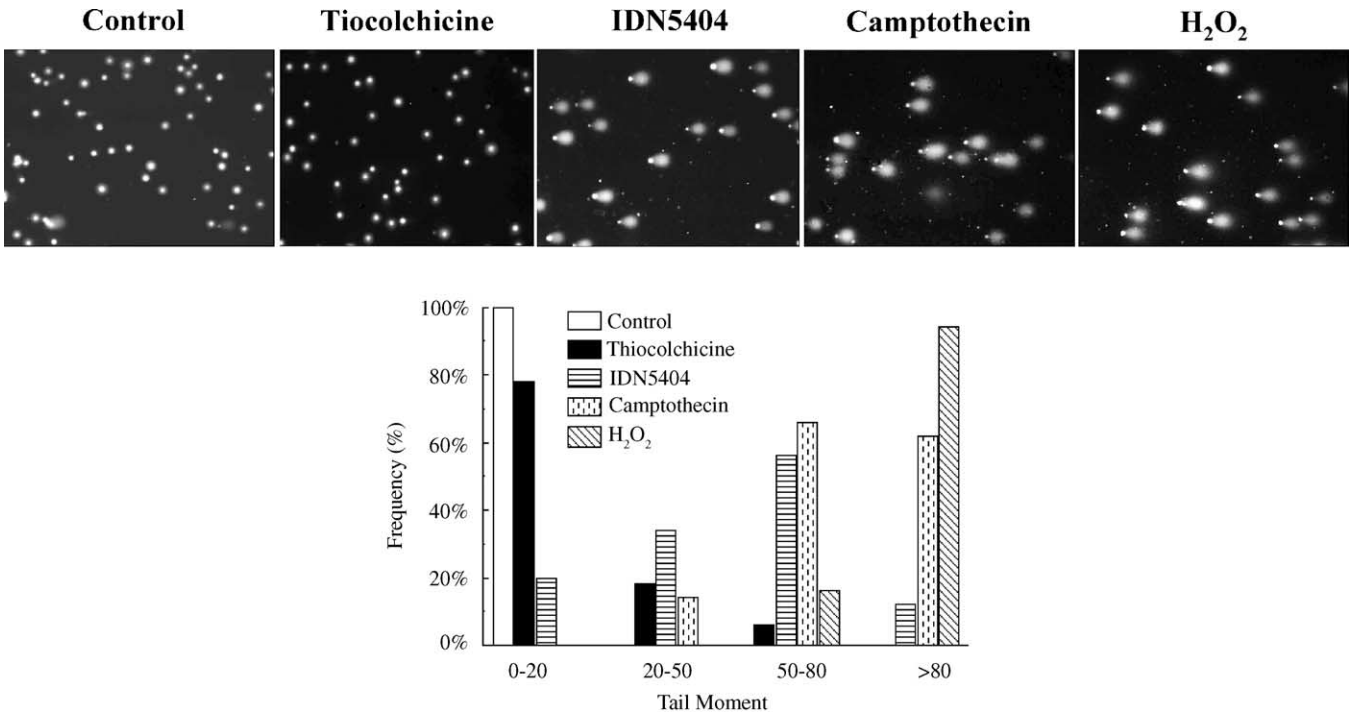

Fig. 3. Comet assay performed in isolated nuclei in the presence of the vehicle (DMSO 0.1%), thiocolchicine (10 μM), IDN5404 (10 μM), CPT (10 μM) or H_2O_2 (50 μM) as positive control. In the insets representative image of the experiment reported in the bar chart. Tail moment for each comet was calculated. Nuclei were grouped according to the tail moment value in four groups and the frequency distribution is shown in the bar chart. DNA damage is increased in nuclei with the highest tail moment values.

assess the possible modulation of DNA repair system upon drug treatment, nuclear expression of the DNA mismatch repair proteins (MLH1, GTBP and MSH2) was investigated. GTBP and MSH2 levels did not change significantly upon drug treatments (data not shown). MLH1 was consistently upregulated in both A2780wt and A2780-CIS (Fig. 2C) by single drug treatment and, even more, by the combination. These results supported the previous

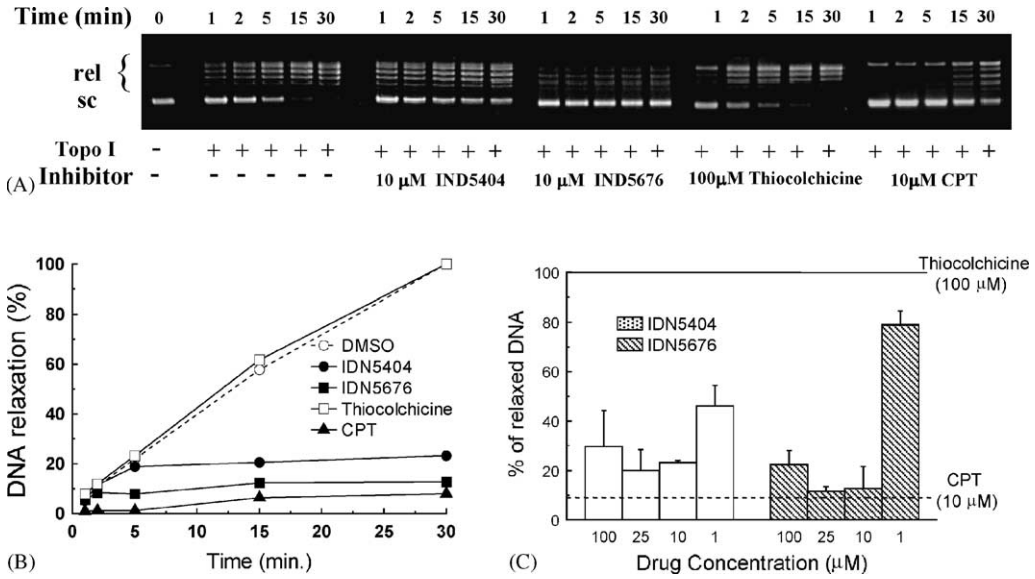

Fig. 4. (A) Agarose gel electrophoresis for a cell free DNA relaxation assay of a supercoiled pBluescript plasmid DNA treated with Topo-I inhibitors from 1 to 30 min. Relaxed DNA topoisomers (rel) slowly migrate in the gel as compared to supercoiled DNA (sc). (B) Line chart showing quantification of the experiment presented in (A). DNA relaxation has been calculated using the ratio between DNA rel/DNA sc. After 30 min the full DNA relaxation is obtained in control lanes, while after 5 min no additional increase of rel DNA is observed in the presence of Topo-I inhibitors. (C) Bar chart showing DNA relaxation activity using two thiocolchicine dimers (IDN5404 and IDN5676) at various concentrations (from 1 to 100 μM). DNA relaxation has been calculated after 30 min. The complete relaxation (100%) is obtained in the absence of Topo-I inhibition. Bars and error bars represent mean and S.D. of three independent experiments, respectively. Dashed and continuous lines indicate the mean of relaxed DNA obtained with thiocolchicine (100 μM) and CPT (10 μM), respectively.

hypothesis that the genotoxic effects of cisplatin are increased by IDN5404, but they do not explain the mechanism(s) by which it occurs.

In order to evaluate a direct involvement in the DNA repair system, freshly isolated cell nuclei were treated for 30 min with the compounds and the occurrence of DNA breaks was assessed by Comet assay (Fig. 3). In Comet assays IDN5404 was able to induce DNA breaks in a manner similar to CPT, a well-known Topo-I inhibitor, whereas the monomer thiocolchicine were unable to produce a similar effect. These experiments revealed that thiocolchicine dimers directly target cell nucleus, independently on a potential interference with the microtubule network.

Cisplatin is a cross-linking agent, maximally inducing intrastrand adducts, rapidly eliminated through the NER pathway and the consequent formation of SSB [11]. Since SSB repair involves Topo-I activity [12] and Topo-I activity is highly enhanced in cisplatin-resistant cells [13], the ability of IDN5404 to modulate Topo-I function was considered. Firstly, we evaluated, in a cell free DNA relaxation assay, whether IDN5404 and IDN5676 inhibited Topo-I relaxation activity of a supercoiled pBluescript plasmid DNA. A representative experiment is shown in Fig. 4A–B, while in Fig. 4C dose–response results coming from three independent experiments are shown. Both dimers IDN5404 and IDN5676 inhibited Topo-I, whereas thiocolchicine did not. As compared to the inhibition induced by 10 μM CPT (91%), IDN5676 and IDN5404 obtained at the same dose inhibition values of 89% and 78%, respectively, thereby indicating that both dimers were able to act as Topo-I inhibitors.

Topo-I acts as a single strand endonuclease and ligase, and CPT inhibits ligase without affecting the cleavage step. Therefore, CPT entraps a slow migrating complex formed by the enzyme the drug and DNA, named as cleavable complex. We assessed whether thiocolchicine dimers also induced the formation of the cleavable complexes, acting as CPT-like compounds (Fig. 5A). Neither IDN5404 nor IDN5676 were able to generate cleavable complexes, thereby indicating that the inhibition of thiocolchicine dimers occurs upstream of the endonuclease activity of Topo-I. Thus, we theorised that dimers could interfere in the binding of Topo-I to DNA. To test this hypothesis, we performed the EMSA between supercoiled pBR322 DNA and Topo-I. We used CPT as a control, which inhibiting the ligase activity does not interfere with the binding of the enzyme to DNA. Results (Fig. 5B) clearly indicated that both dimers hampered the binding of the enzyme to DNA, whereas, as anticipated, this did not occur with thiocolchicine and CPT.

In order to assess the specificity of Topo-I inhibition, we ruled out that thiocolchicine dimers inhibited aspecifically DNase activity and that the compounds were able to intercalate into the DNA. DNase activity was unaffected by drug treatment also at the highest tested concentrations

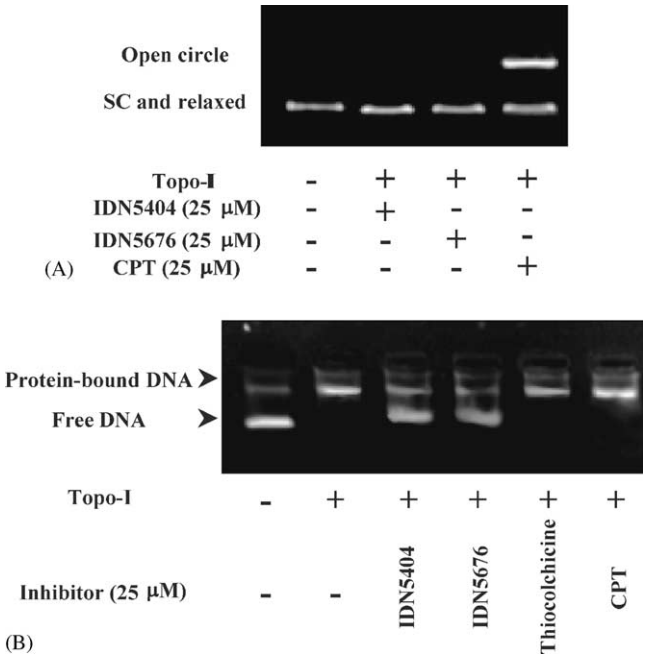

Fig. 5. (A) Representative image of agarose gel electrophoresis of the cleavage assay. PBluescript plasmid DNA was incubated in the presence of an excess of Topo-I (100 U) and with or without Topo-I inhibitors. Reactions were started by addition of DNA and terminated with prewarmed SDS (1%, w/v final concentration). Only CPT induced the formation of a slow migrating complex formed by enzyme, drug and DNA, whereas thiocolchicine dimers IDN5404 and IDN5676 were unable to interfere. This experiment was repeated three times with similar results. (B) Representative image of the agarose gel electrophoresis for EMSA between pBR322 DNA and Topo-I supercoiled pBR322 DNA (Topogen) was incubated without excess of Topo-I (100 U) in the presence of the test compounds at 37 °C for 6 min. The reaction was started by addition of DNA. Samples containing test compounds were assembled in the order compound, Topo-I, Topo-I inhibitor. Thiocolchicine and CPT do not modulate the formation of the complex DNA/Topo-1, whereas both dimers IDN5404 and IDN5676 significantly hamper the binding of the enzyme to DNA. This experiment was repeated three times with similar results.

(Fig. 6A), thus excluding an aspecific inhibition of DNA enzymes. The DNA intercalating ability was assessed through the DNA unwinding assay (Fig. 6B). In fact, intercalating agents are able to produce, after intercalation, supercoiling of DNA structure. Thiocolchicine dimers were unable to modulate DNA unwinding, whereas doxorubicin, used as a positive control, did it. These findings therefore indicate that thiocolchicine dimers are specific inhibitors of Topo-I function and that the mechanism of inhibition is different from that of CPT.

In order to assess if dimers are able to overcome drug-resistance against Topo-I poisons, drugs were tested in two cell lines made resistant in our laboratory to topotecan, the most commonly used CPT analogue. Data shown in Table 1 indicate that both dimers IDN5404 and IDN5676 were able to overcome topotecan resistance.

In order to establish if IDN5404 retained spindle poison activity related to the colchicine structure, cell free tubulin

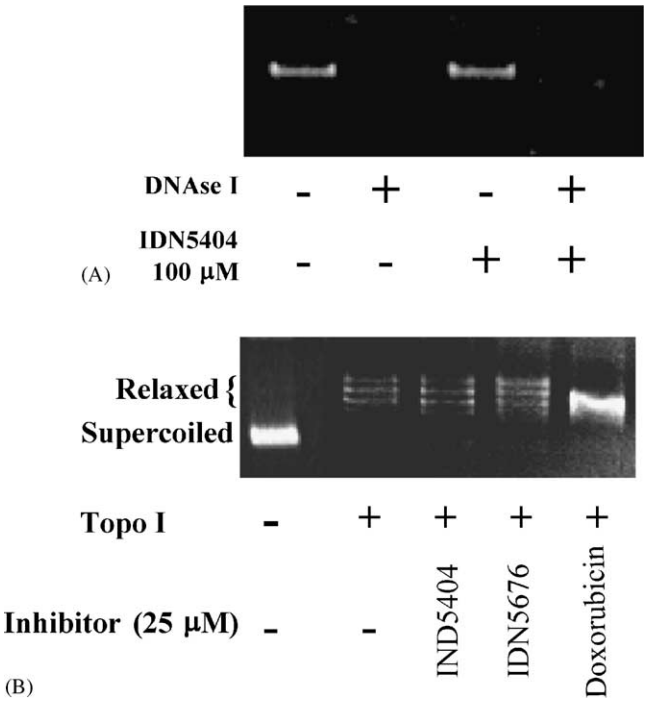

Fig. 6. (A) Representative image of agarose gel electrophoresis of the DNase assay. Bovine DNase I (4.0 U/ml) was incubated with 400 ng of pBR322 DNA in the presence of IDN5404 (up to 100 μM) for 15 min at 37 °C. Thiocolchicine dimer was unable to interfere with DNase activity, thereby indicating the absence of non-specific interactions with DNA. This experiment was repeated three times with similar results. (B) Representative image of the agarose gel electrophoresis for DNA unwinding assay. Supercoiled pBR322 DNA was relaxed with 300 U of Topo-I at 37 °C for 15 min in Topo-I relaxation buffer. Inhibitors were added and the incubations were continued for additional 60 min. Parallel experiments ensured that Topo-I retained its activity in the presence of the compounds used. The reaction was terminated by addition of 1% (w/v) SDS and followed by digestion with proteinase K as described above. Treatment with thiocolchicine dimers did not induce DNA unwinding, thereby pointing out the absence of DNA intercalation. Doxorubicin, a well-known DNA-intercalating agent, was used as positive control and provoked DNA unwinding. This experiment was repeated three times with similar results.

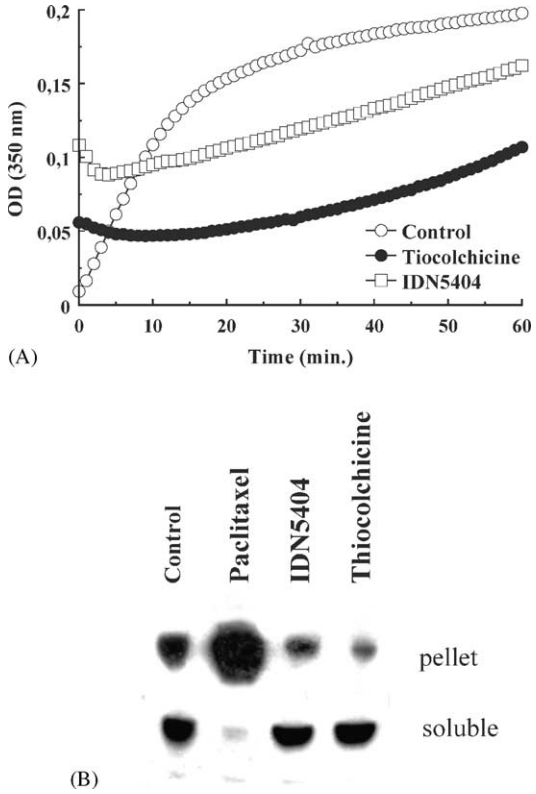

Fig. 7. (A) Line chart reporting results for in vitro tubulin polymerisation assay. The behaviour of IDN5404 as inhibitor of tubulin polymerisation is similar to that of thiocolchicine, albeit at a lower degree of potency. This experiment was repeated three times with similar results. S.D. have been omitted to add clarity to the chart. (B) Representative Western blots for inhibition of microtubule dynamic in whole cells. Cells were exposed to drugs for 30 min and after centrifugation polymerised and free tubulin was separated in the pellet and soluble fraction, respectively. Paclitaxel was used as positive control.

polymerisation assay as well as the activity in whole cells was assessed. In cell free tubulin polymerisation assay IDN5404 was able to produce a depolymerising effect on tubulin polymerisation slightly lower than that of thiocolchicine (Fig. 7A). The same effect was also noticed in whole cells where IDN5404 induced a depolymerising effect on microtubules similar to that of thiocolchicine (Fig. 7B). These experiments confirm that in addition to the Topo-I inhibition IDN5404 retains its activity as a spindle poison and inhibitor of microtubule dynamic.

Table 1
IC_{50} values obtained after 72 h of continuous drug exposure

Drug (nM)	A2780wt	A2780TOP-1	A2780-TOP-2
Topotecan	8.5 ± 3.3	423 ± 212	352 ± 110
IDN5404	18.6 ± 4.2	10.3 ± 7.8	8.2 ± 3.7
IDN5676	5.2 ± 0.7	4.6 ± 0.2	4.6 ± 0.4

Values (±S.D.) are calculated in three independent experiments.

4. Discussion

Thiocolchicine dimers were investigated in this study because during a cellular screening they expressed an increased activity in a cisplatin-resistant cell line. In order to elucidate the molecular basis of the activity in cisplatin-resistant cells, we assessed $p21^{waf1}$, p53 and MLH1 as biological probes for the modulation of DNA-damage induced by cisplatin [14,15] and we found that the thiocolchicine dimer IDN5404 was able to measurably raise levels of $p21^{waf1}$ (only in A2780wt cells), p53 and MLH1 (in both A2780wt and A2780-CIS cells) when used in combination with cisplatin. This finding prompted us to believe that the drug directly interacts with the process of DNA repair stimulated by cisplatin-induced DNA damage.

Cisplatin is a DNA cross-linking agent, able to form prevalently intrastrand cross-link between adjacent purines [16,17]. These DNA adducts are then removed through the NER pathway (reviewed in [11]). In brief, NER-specific damage recognition proteins bind to the DNA in correspondence of the DNA-adduct. Next, the damaged strand is

incised at both sides of the lesion and removed, thereby producing a SSB. Noteworthy, in the absence of a functional NER pathway cisplatin does not produce SSB [18]. Cisplatin-induced SSBs are then repaired and in this process Topo-I plays a prominent role [12]. This mechanism is likely to underlie the increase of Topo-I activity/expression noticed in cisplatin-resistant cells (reviewed in [13]) and the high response rate to Topo-I poisons reported in patients relapsing from the cisplatin treatment [19]. With this in mind, the potential impact of the thiocolchicine dimers in Topo-I function was assessed and fortunately this hypothesis was supported by the discovery that thiocolchicine dimers are strong inhibitors of Topo-I, an activity not shared by thiocolchicine. As opposed to CPT, thiocolchicine dimers did not induce cleavable complexes, thereby indicating that inhibition occurs upstream of the ligase activity of the enzyme. Other naturally derived Topo-I poisons, such as rebeccamycin [20], bulgarein [21] and coralyne [22], inhibit Topo-I through DNA intercalation and specific bonding in the minor groove. Thiocolchicine dimers did not act through this pathway, as demonstrated by the absence of interference in the DNA-unwinding assay. Rather, the mechanism seems very similar to that of some flavones (quercetin, acacetin, apigenin, kaempferol and morin [9]) and acetyl-boswellic acids [23], which are pentacyclic triterpenes derived from the gum resin of frankincense (*Boswellia serrata*). In common with these compounds, thiocolchicine dimers specifically interfere with the binding of Topo-I to DNA, thereby inhibiting the formation of the complex DNA-Topo-I and the subsequent religation step, without entrapping cleavable complexes.

In addition to the Topo-I activity, thiocolchicine dimers retain the ability to interact with tubulin, as demonstrated by the tubulin polymerisation assay in purified tubulin and in whole cells, with a mechanism of action not so different from the parent compound thiocolchicine. The dual mechanism of action could explain an additional feature of thiocolchicine dimers, consisting in the ability to overcome the resistance to the Topo-I poison Topotecan.

Spindle poisons and Topo-I inhibitors are commonly used as antitumour agents. Among the clinically used anticancer drugs, Taxanes and Vinca alkaloids belong to the first category, whereas CPT analogues Topotecan and Irinotecan are in the second. Previous reports have suggested that other synthetic colchicine-derived compounds (demethylcolchiceinamide analogues) interact with DNA and Topo-II [24]. However, demethylcolchiceinamides have a structure very different from those herein reported and such drugs are lacking of the tubulin-interacting properties. Moreover, they do not interfere with Topo-I. A similar dual activity of Topo-I inhibitor and spindle poison has been recently described for the compound BPR0Y007 [25]. However, this compound shares with CPT the same mechanism of Topo-I inhibition, whereas thiocolchicine dimers do not act through the same mechanism and potentially could be useful also in CPT-like resistant cells.

Considering all these findings, thiocolchicine dimers could represent an innovative tool in the fight against drug resistance to conventional antitumour agents such as cisplatin and topotecan. The profile of biological activity warrants ulterior extensive investigations aimed at isolating the pharmacophore responsible for the Topo-I inhibition as well as to characterise the toxicological and antitumour profile in preclinical experimental models.

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